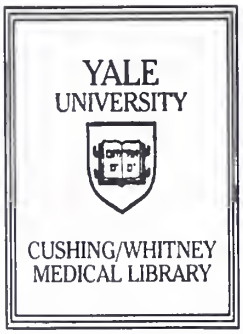
YALE MEDICAL LIBRARY
3 9002 08676 0882
MED
T113
+.Y12
6447

ALTERED EXPRESSION OF P53 AND MDM2 IN SUPERFICIAL BLADDER CANCER

Julian Neville Anthony

Yale University

1997


YALE
UNIVERSITY
CUSHING/WHITNEY
MEDICAL LIBRARY

Permission to photocopy or microfilm processing of this thesis for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Julian Bradshaw

Signature of Author

4/25/97

Date

YALE MEDICAL LIBRARY

AUG 04 1997

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/alteredexpressio00anth

ALTERED EXPRESSION OF P53 AND MDM2 IN SUPERFICIAL BLADDER CANCER

A THESIS SUBMITTED TO THE

YALE UNIVERSITY SCHOOL OF MEDICINE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF MEDICINE

BY

JULIAN NEVILLE ANTHONY

1997

ALTERED EXPRESSION OF P53 AND MDM2 IN SUPERFICIAL BLADDER CANCER.

Julian N. Anthony, Amy Darefsky, and David Esrig. Section of Urology, Department of Surgery, Yale University, School of Medicine, New Haven, CT.

P53 is a tumor suppressor gene shown to be involved in regulation of the cell-cycle. Alterations in the expression and function of p53 have been implicated in a number of human malignancies. Recently a cellular protein, mdm2, has been found to have the ability to bind wild-type p53 and inactivate its function. The purpose of this study was to analyze the frequency of alterations in the tumor markers p53 and mdm2 for superficial bladder cancer. Patient data was compiled from clinical charts of 84 patients at Yale-New Haven and West Haven Veterans Hospitals who underwent transurethral resection for suspected bladder tumors. Tumor specimens were reviewed and the most representative sections were selected for immunohistochemical staining with monoclonal antibodies specific to the p53 and mdm2 proteins. The p53 and mdm2 status was then analyzed in relation to probability of recurrence. P53 nuclear reactivity was detected in 41 of 66 bladder tumors. Mdm2 nuclear reactivity was detected in 8 of 66 bladder tumors. Bladder tumors that were negative for p53 and positive for mdm2 had a rate ratio for tumor recurrence that was 20.02 times that of the reference group (p53 negative, mdm2 negative) (p=0.01). Tumors that were positive for p53 and negative for mdm2 had a rate ratio for tumor recurrence that was 3.25 times that of the reference group (p=0.02). Tumors that were positive for p53 and positive for mdm2 had a rate ratio for tumor recurrence that was 4.00 times that of the reference group (p=0.05). Any aberration in the tumor markers p53 and mdm2 have a rate ratio of tumor recurrence 3.31 times that of the reference group (p=0.01). The expression of mdm2 may function to inactivate wild-type p53 and contribute to tumorigenesis in superficial bladder cancer.

Acknowledgements

I would like to thank my mentor Dr. David Esrig for providing me with the opportunity to undertake this project. I would also like to thank Amy Darefsky, Dr. John Stein, Dr. Matthew Dunn, Sharon Mella, Romula Solon, Critical Technologies, and Biomedical Communications for all of their help and technical support in this study.

I would like to thank and dedicate this manuscript to my mother, Esther Anthony. It has been her emotional guidance and support that has inspired me through medical school and in completion of this thesis. I would also like to thank Azin Shahryarinejad for her continued personal support throughout medical school.

All of the work described in this manuscript was carried out by Julian Anthony with the exception of the statistical analysis which was performed by Amy Darefsky and technical immunohistochemical staining which was performed by Drs. Stein and Dunn and Critical Technologies of the Yale University Pathology Department.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS I

TABLE OF CONTENTS II

FIGURES AND TABLES III

INTRODUCTION 1

METHODS 11

PATIENT POPULATION 11
MONOCLONAL ANTIBODIES AND IMMUNOHISTOCHEMICAL PROCEDURES 11
STATISTICAL ANALYSIS 13

RESULTS 15

TABLE 1: CHARACTERISTICS OF THE STUDY SUBJECTS AND INCIDENT PRIMARY BLADDER TUMORS 22
TABLE 2: THERAPY AND TUMOR MARKER STATUS OF INCIDENT PRIMARY SUPERFICIAL BLADDER TUMORS 23
TABLE 3: THE EFFECT OF P53 AND CONFOUNDING VARIABLES ON THE RATE OF BLADDER CANCER RECURRENCE 24
TABLE 4: THE EFFECT OF P53 ON THE RATE OF BLADDER CANCER RECURRENCE CONTROLLING FOR MDM2 AS A CONFOUNDING VARIABLE 25
TABLE 5: THE EFFECT OF P53 ON THE RATE OF BLADDER CANCER RECURRENCE ASSESSING FOR AN AFFECT MODIFICATION OF P53 BY MDM2 26
TABLE 6: MODELING A CONTRAST: THE PROTECTIVE EFFECT OF (-) P53 AND (-) MDM2 VS. ALL OTHER COMBINATIONS 27
FIGURE 1 IMMUNOHISTOCHEMICAL DETECTION OF P53 PROTEIN IN THE NUCLEI OF TRANSITIONAL-CELL CARCINOMAS OF THE BLADDER WITH THE ANTI-P53 MONOCLONAL ANTIBODY DO7 28
FIGURE 2 IMMUNOHISTOCHEMICAL DETECTION OF MDM2 PROTEIN IN THE NUCLEI OF TRANSITIONAL-CELL CARCINOMAS OF THE BLADDER WITH THE ANTI-MDM2 MONOCLONAL ANTIBODY IF2 29

DISCUSSION 30

BIBLIOGRAPHY 37

FIGURES AND TABLES

FIGURE 1 IMMUNOHISTOCHEMICAL DETECTION OF P53 PROTEIN IN THE NUCLEI OF TRANSITIONAL-CELL CARCINOMAS OF THE BLADDER WITH THE ANTI-P53 MONOCLONAL ANTIBODY DO7 28
FIGURE 2 IMMUNOHISTOCHEMICAL DETECTION OF MDM2 PROTEIN IN THE NUCLEI OF TRANSITIONAL-CELL CARCINOMAS OF THE BLADDER WITH THE ANTI-MDM2 MONOCLONAL ANTIBODY IF2 29

TABLE 1: CHARACTERISTICS OF THE STUDY SUBJECTS AND INCIDENT PRIMARY BLADDER TUMORS 22
TABLE 2: THERAPY AND TUMOR MARKER STATUS OF INCIDENT PRIMARY SUPERFICIAL BLADDER TUMORS23
TABLE 3: THE EFFECT OF P53 AND CONFOUNDING VARIABLES ON THE RATE OF BLADDER CANCER RECURRENCE 24
TABLE 4: THE EFFECT OF P53 ON THE RATE OF BLADDER CANCER RECURRENCE CONTROLLING FOR MDM2 AS A CONFOUNDING VARIABLE 25
TABLE 5: THE EFFECT OF P53 ON THE RATE OF BLADDER CANCER RECURRENCE ASSESSING FOR AN AFFECT MODIFICATION OF P53 BY MDM2 26
TABLE 6: MODELING A CONTRAST: THE PROTECTIVE EFFECT OF (-) P53 AND (-) MDM2 VS. ALL OTHER COMBINATIONS.....27

Introduction

Bladder cancer is the fifth most common cancer in the United States and the second most common cancer of the genitourinary tract with an annual incidence of approximately 18 cases per 100,000[1]. Approximately 51,600 new cases are diagnosed annually with an estimated 9,500 deaths in the United States[2]. 90% of all bladder cancers are transitional cell carcinomas[8]. At initial presentation, approximately 50-70% of bladder tumors are of a superficial nature and low grade consisting of stage Tis (carcinoma in situ) or Ta (confined to the mucosa)[3]. The majority of these patients will have recurrences after transurethral resection of the tumor[4,5,6,7]. For the most part, tumors that present with a low grade and superficial in nature remain so throughout the life of the patient[3]. However, about 25% of these superficially presenting tumors go on to progress in grade[6]. At the time of diagnosis approximately 28% of bladder tumors have invaded the lamina propria (T1) and about 24% have invaded into the muscle wall (>T2)[8]. Metastasis to regional or distant sites are found in approximately 15% of cases[8].

Staging of bladder cancer is dependent upon the depth of invasion. Tis tumors are confined to the mucosal layer. They are described as flat, nonpapillary, high grade, anaplastic epithelium which lacks the normal cellular polarity and whose cells are large and have prominent nucleoli[8]. Ta tumors are confined to the mucosal layer of the urothelium. They are papillary in nature and histologically consist of low grade tumors. T1 tumors extend into the lamina propria of the urothelium. T2

tumors extend into the superficial muscularis propria. T3a tumors extend into the deep muscularis propria. T3b tumors extend into the perivesical fat or peritoneum while T4 tumors extend to contiguous organs such as the prostate or vagina.

Grading of bladder cancer is dependent on a number of histological characteristics. Features such as cell and nuclear size, nuclear-cytoplasmic ratio, nuclear pleomorphism and irregularity, hyperchromasia, chromatin clumping, nuclear crowding and overlapping, prominent nucleoli, and mitotic figures are used to determine grade[9]. The degree of change in these criteria dictate the grade of the tumor ranging from grade one through four.

Recurrence and progression of disease are two important factors in the natural history of bladder cancer[8]. Traditionally criteria such as tumor grade, stage, size, lymphatic invasion, presence of carcinoma in situ, papillary or solid tumor configuration, multifocal tumors, frequency of tumor recurrences and the patient's age have been used in an attempt to predict patterns of recurrence and progression[3]. Bladder tumors confined to the mucosa (Stage Ta) have been shown to have a progression rate of about 3%, those invading the lamina propria (T1) about 25%-33%, and those invading the musculature (>T2) about a 50% rate of progression and metastasis[9]. Tumors with evidence of CIS have a rate of progression and metastasis of about 80%[81]. A current challenge in the treatment of bladder cancer has been to develop biological markers which would better predict disease progression. An accurate biological marker could have a profound effect on current treatment modalities. For example, aggressive therapy could be targeted towards patients more likely to have disease progression.

Tumorigenesis is thought to result from a cell's inability to control proliferation and disorders in the interactions between cells and their surroundings[10]. Oncogenes can cause the formation of tumors when expressed in a cell. They can be activated by a number of genetic processes including mutation, amplification, or rearrangement. Oncogenes are able to manifest their cellular affects through several mechanisms. They can reduce a cells ability to suppress growth and respond to growth control signals through growth factors and receptors involved with signal transduction pathways. Oncogenes may also code for transcription factors which regulate the expression of genes involved in control of the cell cycle[2,3].

Tumor suppressor genes (TSGs) can inhibit a tumor cell's ability to grow. Tumor suppressor genes act in a recessive manner in that both alleles must be lost or inactivated in order to lose growth inhibition. However, some tumor suppressor genes may appear to behave in a dominant fashion like an oncogene. This usually results from one allele being constitutionally deleted or an abnormal gene product interfering with the function of the normal one. For example in the hereditary form of retinoblastoma, the offspring inherits one abnormal gene. A subsequent somatic mutation to the remaining normal allele results in cancer[2,3].

Theories regarding the precise mechanisms of action of TSGs are constantly evolving based on continually expanding experimental data. TSG products may bind and inactivate transcription factors which activate genes involved in the progression of the cell cycle. Conversely, TSGs may code for transcription factors which activate genes involved in inhibition of the cell cycle[2].

A number of TSGs have been identified which may play a role in the development and progression of bladder cancer. The retinoblastoma (RB) susceptibility gene codes for a 110 kD nuclear phosphoprotein thought to be involved in regulation of the cell cycle. A number of independent investigators have shown that alterations in the expression of the RB protein may be associated with more aggressive muscle-invasive bladder tumors and may be a prognostic indicator of tumor progression[63-67].

Several oncogenes also exist which are thought to play a role in the development and progression of bladder cancer. Epidermal growth factor (EGF) is a protein mitogen that is excreted in urine in high concentrations in a biologically active form[68]. EGF most likely initiates its tumorigenic effects through binding with its receptor (EGF-R) and subsequent growth stimulation[69,70] and induction of ornithine decarboxylase activity[69]. Messing has shown there to be an increased concentration of receptors in premalignant and malignant urothelium[71]. Both Berger et al., and Wright et al., have shown that the overexpression of EGF-Rs on urothelium correlates with tumor grade and stage in transitional cell carcinomas of the bladder[72,73]. The protooncogene c-src is involved in differentiation of the nervous system[74-76] and neoplastic progression of colon carcinomas[77,78]. A study by Fanning et al., correlated expression of the c-src protooncogene with histological grade of bladder carcinomas and suggested a role for c-src in the differentiation of urothelial cells[79]. The ras gene family is involved in a number of different human malignancies[80]. The H-ras gene product is a 21 kD cell membrane protein involved in signal transduction pathways[81]. A recent report by Ooi et al., found there to be a

high frequency of H-ras mutations in transitional cell carcinomas of the bladder[82]. Viola et al., found there to be increased expression of the H-ras gene product, p21, in high grade tumors and in premalignant lesions thus suggesting that overexpression of p21 may be a prognostic indicator in transitional cell carcinoma of the bladder[83].

There is a plethora of experimental data implicating p53 as a TSG. When the wild-type p53 gene is cotransfected into primary rat embryo fibroblasts with activated oncogenes the transformation frequency is decreased[25,26]. Additionally, studies by Chen et al. demonstrated a decrease in the tumorigenic potential of transformed cells with the addition of p53 cDNA[27]. Abnormalities of the p53 gene have been observed within a broad spectrum of human malignancies involving lung, breast, stomach, bladder, testis, bone, and colorectum[11].

The p53 gene is a tumor suppressor gene that has been mapped to human chromosome 17p13.1. Tools of genetic analysis such as cytogenetic and restriction fragment length protein (RFLP) studies have found aberrations in chromosome 17 associated with human malignancies. Allelic deletions of chromosome 17p have been found in approximately 60% of tumors involving the colon, breast, lung, ovaries, cervix, adrenal cortex, and bone and in at least 30% of brain tumors[12-18]. The p53 gene contains 11 exons and 10 introns. Exons 2-11 code for the actual p53 protein. However, exons 5-9 are evolutionarily highly conserved regions and contain the majority of genetic mutations. The majority, greater than 95%, of these mutations are base substitutions and have been localized to four of the five evolutionarily conserved domains[19]. It has also been shown that mutations of specific codons have been associated with different cancer types[20,21].

A number of studies have established a correlation between overexpression of the p53 protein and prognosis in patients with bladder cancer. Esrig et al., were able to show that overexpression of p53 in tumor cell nuclei predicts a significantly increased risk of recurrence and death[54]. Lipponen found overexpression of p53 protein to be related to progression of stage in bladder cancer. He also found overexpression of p53 protein to be a significant predictor of survival in papillary and muscle-invasive bladder tumors[55]. Sarkis et al., have a number of studies correlating overexpression of p53 with prognosis and survival in different stages of bladder cancer. In one study, they found that T1 bladder cancers that overexpress p53 protein have a higher probability of disease progression and that p53 overexpression is an important prognostic factor[56]. Subsequent work by Sarkis et al., found that patients with stage Tis disease and p53 nuclear overexpression had a significantly higher probability for disease progression and death from bladder cancer[57]. Another study showed p53 nuclear overexpression to be an independent prognostic indicator for survival in patients with invasive bladder cancer[58].

The p53 protein has been shown to have site specific DNA binding capacity. It is the central portion of the protein, encoded by the DNA exons with the highest mutational frequency, that is responsible for this DNA binding activity[22]. P53 has been shown to stimulate and inhibit gene expression most likely through its DNA binding capacity as a transcriptional factor. Codons 20-42 code for the acidic activation domain responsible for this activity[23]. Work by Mack et al. illustrates p53's inhibitory effects on gene expression, more specifically on genes with a TATA promotor region[24].

Maltzman et al. and Kastan et al. demonstrated that increases in cellular levels of p53 occur after exposure to DNA damaging elements such as UV light, gamma irradiation, and chemotherapeutic agents[28,29]. Kuerbitz et al. were able to show that these increased levels of p53 resulted in arrest of the cell-cycle at the G1-S phase interface[30]. P53 is thought to function as the "molecular policeman" of the genome. P53 induces the cell to arrest prior to the DNA replication of S phase. In this manner, mutations of the genome are not propagated down further cell lines. If repair of the damaged DNA is not possible, p53 is thought to induce apoptosis (programmed cell death)[31,32]. The precise mechanisms of how p53 induces apoptosis are currently unknown. However, there is genetic evidence indicating how p53 induces arrest of the cell cycle. The wild- type p53-activated fragment (WAF-1) protein may play a role in p53 induced cell-cycle arrest. The WAF-1 gene is a p53 inducible gene[33]. WAF-1 protein (p21) forms a complex with cyclin-dependent kinases (CDK's) and inhibits their activity[19]. CDK's are enzymes involved in the progression of the cell cycle. Therefore, WAF-1 has the ability to arrest the cell cycle through CDK inactivation.

Recently, molecular genetic studies have allowed for more detailed analysis of genomic function in addition to the established assessment of genomic integrity. With improvements in immunohistochemistry (IHC), we are now able to assess function of the p53 gene at the protein level. Alterations of the p53 gene consist of point mutations, allelic losses, rearrangements, deletions, and germline mutations[20]. A number of molecular studies have been utilized in the characterization of these mutations including direct sequencing of PCR amplified DNA fragments and single

strand conformational polymorphism analysis (SSCP)[19]. The sensitivity of SSCP is >99% for 100-300 bp fragments, however the mutated sequence must be at least 3-5% of the DNA mixture[34,35]. Other disadvantages of these molecular analyses are that they are very cumbersome and time consuming and not very practical for everyday clinical use. Recently immunohistochemical (IHC) techniques have been used in the detection of overexpressed p53 protein. Wild-type p53 has a relatively short half-life of 5-40 minutes and its nuclear presence is usually not detectable via immunohistochemical procedures[36]. However, mutations in the p53 gene lead to conformational changes in protein structure and increased stability of the protein with a resultant increased half-life of many hours[37]. It is the p53 protein with increased stability that is detected by IHC. Studies by Esrig et al., have shown there to be a good correlation between overexpression of p53 (detected by IHC) and mutations in the p53 gene (detected by molecular genetic analysis)[36]. However, false positives and false negatives still persist which can be explained by a number of hypotheses(See discussion). IHC analysis may be more sensitive than genetic molecular studies. IHC is able to detect protein expression on a cell to cell basis whereas the sensitivity of molecular analysis depends upon the ratio of mutant to wild-type DNA. IHC is also able to assess the functional status of the p53 protein. It is the nonfunctioning protein with increased stability that is detected by IHC. Whereas with genetic analysis there are several scenarios in which a normal gene may be present but the protein may be nonfunctioning secondary to the cellular environment (see below).

P53 has been shown to form oligomeric protein complexes with a number of viral and cellular proteins[19]. P53 was originally discovered as a complex bound to the simian virus 40 (SV40) large-T antigen[38,39] and subsequently was found to form complexes with human papillomavirus E6[40], Epstein-Barr virus nuclear antigen 5[41], adenovirus type 5 E1b 55-kDa protein[42], hepatitis B virus X antigen[43], and lymphotropic papovavirus large tumor antigen[44]. The functional status of p53 is determined by a number of factors including its physical conformation, cellular environment, and interactions with cellular and viral proteins[19]. As discussed above, many of the genetic mutations involving the p53 gene lead to conformational changes in protein structure which alters the overall functional capacity and stability of p53. Levine et al., demonstrated how different mutations in the p53 gene result in proteins with different functional capacities[45]. For example, a mutated p53 protein with a substituted amino-acid residue 175 leads to loss of its transactivation capability[45]. Protein stability, as measured by half-life, is also affected by cellular and viral protein interactions. Sarnow et al., demonstrated that p53 has a much longer half-life when complexed with the viral proteins SV40 large T antigen and adenovirus E1b 55kDa protein [42,46]. Conversely, binding of p53 to the human papillomavirus types 16 and 18 E6 protein rapidly increases its rate of degradation[40]. A number of independent investigators have shown p53 loses its transactivation capability when bound to specific viral proteins[38,39,40,42]. Based on the above findings, IHC has proven more sensitive in the assessment of p53 function than genetic molecular analysis. IHC has the advantage of being able to detect accumulated wild-type p53 whose function is altered through viral protein binding.

Mdm2 (murine double minute-2) is a gene that encodes for a 90 kDa protein which has the ability to bind p53 and inactivate its activity as a transcriptional factor[47]. The human MDM2 gene has been localized to chromosome 12q 13-14[48]. The MDM2 protein has been shown by Oliner et al., to possess the ability to bind p53 and conceal its activation domain from cellular transcription elements[49]. MDM2 was first discovered as an amplified gene on a double minute chromosome of the spontaneously transformed mouse cell line BALB/c[50]. Oliner et al., also demonstrated that MDM2 gene amplification is associated with human sarcomas of soft tissue and bone[48]. On a cellular level MDM2 gene amplification and overexpression increases the tumorigenic potential of cells[51,52]. Work by Wu et al., has shown there to be an autoregulatory feedback loop between p53 and MDM2[53]. They were able to show that the MDM2 gene contains a p53 DNA binding site and that expression of this gene could be regulated by wild-type p53[53]. Furthermore, the MDM2 protein is able to inhibit p53 function as described above. So, MDM2 expression is regulated by p53 at the transcriptional level while p53 activity is regulated by MDM2 at the protein level.

The purpose of this study was to analyze the frequency of abnormalities in p53 and MDM2 expression for superficial bladder cancer. We also hope to determine if alterations in either may be of any clinical significance in terms of indicators of recurrence or potential prognostic factors.

Methods

Patient Population

I reviewed the medical charts of patients at the Yale-New Haven Hospital and West Haven VA who presented for transurethral resection of suspected bladder tumors between January 1989 and January 1996. Patients were selected whose biopsy specimens revealed superficial disease consisting of stages Ta, T1, and Tis. The patients clinical information was compiled consisting of age, sex, presenting symptoms, diagnostic work up, tumor grade, stage, multifocality, presence of carcinoma in situ, treatment received and subsequent follow up. Sections of the paraffin embedded biopsy specimens stained with hematoxylin-eosin were reviewed and the most representative tumor specimen was selected for sectioning and immunohistochemistry with the appropriate antibody.

Monoclonal Antibodies and Immunohistochemical Procedures

Five micrometer sections of archival formalin-fixed, paraffin-embedded tissue were placed on Silane coated slides and heated at approximately 60°C for one hour. Deparaffinization was accomplished with iodine-xylene and tissue was rehydrated with 95% ethanol. Endogenous peroxidase was quenched in 3% hydrogen peroxide/methanol. For staining with the anti-p53 antibody, antigen retrieval was performed with a citrate buffer (pH 6) for a total of 10 minutes (two separate 5 minute intervals) in a standard microwave unit. Tissue was then cooled and incubated with a 5% horse serum for blocking purposes. The anti-p53 mouse

monoclonal antibody D0-7 (DAKO-p53 Protein; dilution, 1:200, DAKO Japan, Kyoto, Japan) was applied to tissue for overnight incubation at 4°C. The exact binding site of the D07 antibody is currently unknown. Phosphate-buffered saline was used to wash the tissue followed by incubation with a biotinylated horse anti-mouse secondary antibody at a concentration of 1:200 (Vector Labs, Burlingame, Calif.). Visualization of reactivity with an avidin-biotin complex immunoperoxidase system (Vector) according to the manufacturer's recommendations was followed by diamino-benzidine (0.03%) used as a chromogen and hematoxylin for the counterstain. Samples of bladder carcinoma with known p53 mutations and documented accumulation of p53 protein by immunohistochemical analysis were used as positive controls. Normal urothelium and nonepithelial cells (lymphocytes, stromal cells, and endothelial cells), used as internal negative controls, demonstrated no immunoreactivity. Only nuclear localization of immunoreactivity was evaluated.

The extent of nuclear reactivity was classified in four categories. No nuclear reactivity; a few focally positive nuclei (1 to 9 percent of tumor cells); heterogenous nuclear reactivity (10 to 49 percent of tumor cells); and intense homogeneous nuclear reactivity (50 to 100 percent of tumor cells) (Fig. 1). Only samples demonstrating at least 10 percent nuclear reactivity were considered to be p53-positive (to have an alteration in p53). Tissue samples demonstrating less than 10 percent nuclear reactivity were considered p53 negative. This criterion was based on the demonstration of a strong correlation of mutations in the p53 gene with the accumulation of p53 protein in 10 percent or more of the tumor-cell nuclei[36]. All cases were independently read by two investigators (JNA, DE). The

immunohistochemical analysis was performed without knowledge of the tumor stage or the results of clinical follow-up.

For the anti-mdm2 antibody, antigen retrieval consisted of a 10 mM sodium citrate buffer (pH 6.0) heated to 95°C in a water bath for 10 minutes. Otherwise the procedure for mdm2 staining was the same as for p53. The anti-mdm2 mouse monoclonal antibody IF2 (IgG2b class, at concentration 5.0 ug/ml, Oncogene Science, Uniondale, N.Y.) was applied to tissue for overnight incubation at 4°C. IF2 recognizes an epitope in the amino terminal portion of the 491 amino acid human mdm2 protein. The OSA-cl cell line known to overexpress the mdm2 protein[59] was used as a positive control and in determining the concentration of the anti-mdm2 antibody. Serial dilutions were used to minimize background staining and achieve optimal nuclear staining. Normal urothelium and nonepithelial cells (lymphocytes, stromal cells, and endothelial cells), used as internal negative controls, demonstrated no immunoreactivity. Only nuclear localization of immunoreactivity was evaluated. The nuclear reactivity criterion described above for the p53 staining was also used for evaluation of mdm2 staining (Fig. 2).

Statistical Analysis

To allow for the variable length of follow up, survival analysis was employed. Cox proportional hazards regressions were performed to assess the dependence of failure time (time to recurrence) on p53 status, controlling for potential confounders and exploring effect modification by mdm2 status. The rate ratio was used to summarize the strength of association between the potential risk factors and the risk

of a recurrent tumor. The term censored describes patients who did not have a recurrence of their tumor or who were lost to follow up during the study. The validity of the Cox regressions was assessed to ensure that the underlying assumption of constant hazard rate ratios was not violated. For the purpose of the statistical analysis, nuclear accumulations of p53 and mdm2 were classified as either positive (accumulation in 10 percent or more of tumor cells) or negative.

Results

Tables 1 and 2 summarize characteristics of the subjects studied and their incident bladder tumors. The study consisted of 84 patients and 85 incident bladder tumors with one subject presenting with two distinct tumors. The mean age of the subjects was 72.9 (range 43 to 90) years. 78 (93%) of the subjects were male and 6 (7%) were female. 35 (42%) were patients from the Veterans Hospital and 49 (58%) were from Yale-New Haven Hospital. Of the 85 bladder tumors 8 (9%) were classified histologically as grade 1, 43 (51%) as grade 2, 31(36%) as grade 3, and 3 (4%) as grade 4. 54 (64%) of the tumor specimens were a pathologic stage Pa (confined to the mucosa), 23 (27%) were P1 (superficial lamina propria invasion), and 7 (8%) were Pis (carcinoma in situ). 16 (19%) of the subjects had biopsy proven carcinoma in situ. Of the 84 patients, 28 (33%) received some form of intravesical chemotherapy. Secondary to incomplete charts or inadequate tissue specimens, data was missing for stage (n=1), p53 status (n=8), and mdm2 status (n=18). As a result of this missing data our sample size analyzed included 66 patients and 66 tumors. The sample size analyzed was a subset of the total sample population and did not possess any significant different characteristics from the total sample population. Of the 66 subjects analyzed, 41 (62%) were positive for overexpression of the p53 protein. 8 (12%) of the 66 subjects were positive for overexpression of the mdm2 protein. 40 (61%) of the 66 patients analyzed had a recurrence of their tumor. The mean

number of months until last follow up for censored patients was 23.4 (range 0 to 62) while the mean number of months until recurrence was 9.23 (range 1 to 40).

Tables 3 through 5 summarize the effect of modeling mdm2 in multivariate models with p53. A multivariate model was constructed to examine the effect of different variables on the rate of recurrence for superficial bladder cancer. The model included all variables which in and of themselves contributed significantly to predicting bladder cancer recurrence and any variable which influenced p53's ability to predict bladder cancer recurrence (a confounder). Variables predictive of bladder cancer recurrence were selected based on significant p-values (<.05). Variables influencing p53's ability to predict bladder cancer recurrence (confounders) were selected based on their ability to cause at least a 10 percent change in the parameter estimate which the rate ratio is based upon. The confounders and variables predictive of bladder cancer recurrence included stage, presence of CIS (carcinoma in situ) and whether or not chemotherapy was initiated. Table 3 models the effect of p53 and confounding variables on the rate of bladder cancer recurrence. Table 4 models the effect of p53 on the rate of bladder cancer recurrence controlling for mdm2 as a confounding variable. Table 5 models the effect of p53 on the rate of bladder cancer recurrence assessing for an effect modification of p53 by mdm2 and takes into account the status of mdm2 (positive versus negative). Table 3 states that the rate of recurrence for bladder tumors that were p53 positive is 2.70 times that of tumors that were p53 negative. The 95% confidence intervals were from 1.10 to 6.61 with a significant p-value of 0.03. These values controlled for stage, presence of CIS, and chemotherapy. Table 3 also modeled the effect of stage, presence of CIS, and

chemotherapy on the rates of recurrence. Pathological stage Pis had a rate of recurrence 6.04 times that of pathological stage Pa. The 95% confidence intervals were from 1.13 to 32.45 with a p-value of 0.04. The presence of CIS had a rate of recurrence 4.89 times that of tumors with no evidence of CIS. The 95% confidence intervals were from 1.35 to 17.68 with a p-value of 0.02. Having received intravesical chemotherapy correlated with a rate of recurrence 0.32 times that of patients that did not receive chemotherapy. The 95% confidence intervals were from 0.10 to 1.07 with a borderline significant p-value of 0.07. Having received chemotherapy was one of the variables that when removed from our model it significantly effected the predictive value of p53 in tumor recurrence. The Likelihood Ratio (LR) Chi-square test was used to assess if the model as a whole was significant. The LR Chi-square was 14.39 with 5 degrees of freedom and a p-value of 0.01 for our model of p53 alone in table 3. The LR test of significance was also used to assess significance of the model as a whole and was 5.4 with 1 degree of freedom and a p-value of 0.02 for the model of p53 alone in table 3.

Table 4 models the same variables as in table 3 with the addition of mdm2 as a confounding factor. The rate ratio of bladder tumor recurrence for p53 positive tumors was 2.62 times that of p53 negative tumors with 95% confidence intervals of 1.06 to 6.49 and a p-value of 0.04. These values controlled for stage, presence of CIS, chemotherapy and mdm2 as confounding factors. Mdm2 positive tumors did not have a significantly increased rate ratio for tumor recurrence when compared to mdm2 negative tumors. The rate ratio was 1.52 with 95% confidence intervals between 0.56 and 4.08 and a p-value of 0.41. With mdm2 included as a confounding

factor, bladder tumors that were positive for p53 and negative for mdm2 had a rate ratio for tumor recurrence that was 2.62 times that of the reference group (p53 negative, mdm2 negative) with 95% confidence intervals from 1.06 to 6.49 and a p-value of 0.04. Tumors that were positive for p53 and positive for mdm2 had a rate ratio for tumor recurrence that was 3.97 times that of the reference group with 95% confidence intervals from 1.11 to 14.29 and a p-value of 0.03. Table 4 also modeled the effect of stage, presence of CIS, and chemotherapy on the rates of recurrence. Pathological stage Pis had a rate of recurrence 6.27 times that of pathological stage Pa. The 95% confidence intervals were from 1.16 to 33.99 with a p-value of 0.03. The presence of CIS had a rate of recurrence 5.30 times that of tumors with no evidence of CIS. The 95% confidence intervals were from 1.43 to 19.62 with a p-value of 0.01. Having received intravesical chemotherapy correlated with a rate of recurrence 0.30 times that of patients that did not receive chemotherapy. The 95% confidence intervals were from 0.09 to 1.00 with a p-value of 0.05. The LR Chi-square was 15.02 with 6 degrees of freedom and a p-value of 0.02 for our model of p53 and mdm2 in table 4. The LR test of significance was also used to assess significance of the model as a whole and was 6.02 with 2 degrees of freedom and a p-value of 0.05 for the model of p53 and mdm2 in table 4. The LR test of significance was used to assess if the current model added any significant information regarding the rates of recurrence when compared to the previous model. Compared to the previous model of p53 alone, the LR Chi-square was 0.62 with one degree of freedom and a nonsignificant p-value of 0.431. This demonstrates that our model with the addition of mdm2 as a confounding factor added no significant

information regarding the rates of recurrence when compared to our model of p53 alone.

Table 5 models the effect of p53 on the rate of bladder cancer recurrence assessing for an affect modification of p53 by mdm2. The rate ratio of tumor recurrence for p53 positive subjects was 3.25 times that for p53 negative subjects when mdm2 was negative. The 95% confidence intervals were from 1.25 to 8.47 with a p-value of 0.02. The rate ratio of tumor recurrence for p53 positive subjects was 0.20 times that for p53 negative subjects when mdm2 was positive. The 95% confidence intervals were from 0.02 to 2.11 with a non-significant p-value of 0.18. The rate ratio of tumor recurrence for mdm2 positive subjects was 20.02 times that for mdm2 negative subjects when p53 was negative. The 95% confidence intervals were from 1.81 to 221.72 with a p-value of 0.01. The rate ratio of tumor recurrence for mdm2 positive subjects was 1.23 times that for mdm2 negative subjects when p53 was positive. The 95% confidence intervals were from 0.42 to 3.63 with a non-significant p-value of 0.70. Bladder tumors that were negative for p53 and positive for mdm2 had a rate ratio for tumor recurrence that was 20.02 times that of the reference group (p53 negative, mdm2 negative) with 95% confidence intervals from 1.81 to 221.72 and a p-value of 0.01. Tumors that were positive for p53 and negative for mdm2 had a rate ratio for tumor recurrence that was 3.25 times that of the reference group with 95% confidence intervals from 1.25 to 8.47 and a p-value of 0.02. Tumors that were positive for p53 and positive for mdm2 had a rate ratio for tumor recurrence that was 4.00 times that of the reference group with 95% confidence intervals from 1.01 to 15.94 and a p-value of 0.05. Table 5 also modeled

the effect of stage, presence of CIS, and chemotherapy on the rates of recurrence. Pathological stage Pis had a rate of recurrence 6.73 times that of pathological stage Pa. The 95% confidence intervals were from 1.19 to 38.23 with a p-value of 0.03. The presence of CIS had a rate of recurrence 6.97 times that of tumors with no evidence of CIS. The 95% confidence intervals were from 1.77 to 27.40 with a p-value of 0.01. Having received intravesical chemotherapy correlated with a rate of recurrence 0.26 times that of patients that did not receive chemotherapy. The 95% confidence intervals were from 0.07 to 0.94 with a p-value of 0.04. The LR Chi-square was 18.15 with 7 degrees of freedom and a p-value of 0.01 for our model of p53, mdm2 and their interaction in table 5. The LR test of significance was also used to assess significance of the model as a whole and was 9.15 with 3 degrees of freedom and a p-value of 0.03 for the model of p53, mdm2, and their interaction in table 5. The LR test of significance was used to assess if the current model added any significant information regarding the rates of recurrence when compared to the previous model. Compared to the previous model of p53 and mdm2, the LR Chi-square was 3.14 with one degree of freedom and a significant Wald p-value of 0.03. This demonstrates that our model with the addition of mdm2 assessing for an interaction with p53 did add significant information regarding the rates of recurrence when compared to our previous model of p53 and mdm2.

Table 6 summarizes the protective effect of negative p53 and negative mdm2 versus all other combinations. Column 1 (p53, mdm2, and interaction) is described in table 5. Column 2 (contrast) combines all tumor marker combinations (p53-mdm2+; p53+ mdm2 -; p53+ mdm2+) and compares them to the reference group of

p53 negative, mdm2 negative. Any aberration in the tumor markers p53 and mdm2 have a rate ratio of tumor recurrence 3.31 times that of the reference group (p53 negative, mdm2 negative) with 95% confidence intervals from 1.29 to 8.49 and a p-value of 0.01. As in all previous models, column 2 also modeled the effect of stage, presence of CIS, and chemotherapy on the rates of recurrence. Pathological stage Pis had a rate of recurrence 6.77 times that of pathological stage Pa. The 95% confidence intervals were from 1.22 to 37.65 with a p-value of 0.03. The presence of CIS had a rate of recurrence 5.73 times that of tumors with no evidence of CIS. The 95% confidence intervals were from 1.57 to 20.92 with a p-value of 0.01. Having received intravesical chemotherapy correlated with a rate of recurrence 0.30 times that of patients that did not receive chemotherapy. The 95% confidence intervals were from 0.09 to 1.02 with a p-value of 0.05. The LR Chi-square was 16.30 with 5 degrees of freedom and a p-value of 0.01 for our model of contrast in column 2. The LR test of significance was also used to assess significance of the model as a whole and was 7.30 with 1 degree of freedom and a p-value of <0.01 for the model of contrast in column 2.

Table 1: Characteristics of the study subjects and incident primary superficial bladder tumors.

		Total Sample 84 patients, 85 tumors		Sample Analyzed known p53, mdm2, followup months 66 patients, 66 tumors	
Study Subjects					
Age at incidence		Mean 72.9 Q: 43,67.5,73,78,90		Mean 72.6 Q: 43,68,73,78,90	
		n	%	n	%
Sex	male	78	93	61	92
	female	6	7	5	8
Hospital	VA	35	42	31	47
	YNHH	49	58	35	53
Primary Tumor Characteristics					
Grade	G1	8	9	6	9
	G2	43	51	35	53
	G3	31	36	22	33
	G4	3	4	3	5
Stage*	Ta	54	64	43	65
	T1	23	27	19	29
	Tis	7	8	4	6
CIS	positive	16	19	11	17
	negative	69	81	55	83

* Data were missing for Stage (n=1), p53 status (n=8), and mdm2 status (n=18).
Q: represents minimum, first quartile, median, third quartile and maximum value.

Table 2: Therapy and tumor marker status of incident primary superficial bladder tumors.

	Total Sample 84 patients, 85 tumors		Sample Analyzed known p53, mdm2, followup months 66 patients, 66 tumors	
	n	%	n	%
Chemotherapy initiated				
no	57	67	41	62
yes	28	33	25	38
p53*	44	57	41	62
positive	33	43	25	38
negative				
mdm2*	8	12	8	12
positive	59	88	58	88
negative				
Recurrences				
Censored	34	40	26	39
Recurred	50	60	40	61
Months to last f/u if cens.	Mean 22.6 Q: 0,6,22,32,62		Mean 23.4 Q: 0,6,24,32,62	
Months to recurrence	Mean 9.26 Q: 1,4,6.5,11,40		Mean 9.23 Q: 1,3.5,6.5,11,40	

* Data were missing for Stage (n=1), p53 status (n=8), and mdm2 status (n=18).
Q: represents minimum, first quartile, median, third quartile and maximum value.

Table 3: The effect of p53 and confounding variables on the rate of bladder cancer recurrence for sample analyzed

		p53 only		
Tumor markers:		RR	95% CI	Wald p-value
p53	pm vs pm			
(+) vs. (-)		2.7	(1.10, 6.61)	0.03 s
If mdm2 (-)	+ - vs - -			
If mdm2 (+)	+ + vs - +			
mdm2				
(+) vs. (-)				
If p53 (-)	- + vs - -			
If p53 (+)	+ + vs + -			

	p53	mdm2
24	-	-
1	-	+
34	+	-
7	+	+

Control variables:				
Stage	Ta	1	Ref	
	T1	1.59	(0.54, 4.68)	0.4
	TIS	6.04	(1.13, 32.45)	0.04 s
CIS	yes vs. no	4.89	(1.35, 17.68)	0.02 s
Chemotherapy	yes vs. no	0.32	(0.10, 1.07)	0.07 b

Significance tests:	LR Chi-square	df	p-value
Model: LR Chi-square, df, p-value	14.39	5df	0.01 s
LR test of significance: Of tumor markers vs previous model	5.4	1df	0.020 s

b=borderline significant at .10 level (<.10,>=.05); s=significant at .05 level (<.05,>=.01); h=highly significant at .01 level (<.01); p=p53, m=mdm2, RR=rate ratio, df=degrees of freedom, CI=confidence intervals, (-)=negative, (+)=positive

Table 4: The effect of p53 on the rate of bladder cancer recurrence controlling for mdm2 as a confounding variable for sample analyzed.

		p53 and mdm2		
Tumor markers:		RR	95% CI	Wald p-value
p53	pm vs pm			
(+) vs. (-)		2.62	(1.06, 6.49)	0.04 s
If mdm2 (-)	+ - vs - -			
If mdm2 (+)	+ + vs - +			
mdm2				
(+) vs. (-)		1.52	(0.56, 4.08)	0.41
If p53 (-)	- + vs - -			
If p53 (+)	+ + vs + -			
	p53 mdm2			
24	- -	1	Ref	
1	- +	1.52	(0.56, 4.08)	0.41
34	+ -	2.62	(1.06, 6.49)	0.04 s
7	+ +	3.97	(1.11, 14.29)	0.03 s
Control variables:				
Stage	Ta			
	T1	1.66	(0.56, 4.90)	0.36
	TIS	6.27	(1.16, 33.99)	0.03 s
CIS	yes vs. no	5.3	(1.43, 19.62)	0.01 s
Chemotherapy	yes vs. no	0.3	(0.09, 1.00)	0.05 b
Significance tests:		LR Chi-square	df	p-value
Model: LR Chi-square, df, p-value		15.02	6df	0.02 s
LR test of significance:				
Of tumor markers		6.02	2df	0.049 s
vs previous model		0.62	1df	0.431

b=borderline significant at .10 level (<.10,>=.05); s=significant at .05 level (<.05,>=.01); h=highly significant at .01 level (<.01); p=p53, m=mdm2, RR=rate ratio, df=degrees of freedom, CI=confidence intervals, (-)=negative, (+)=positive

Table 5: The effect of p53 on the rate of bladder cancer recurrence assessing for an affect modification of p53 by mdm2 for sample analyzed.

		p53, mdm2 and interaction		
Tumor markers:		**RR**	**95% CI**	**Wald p-value**
p53	pm vs pm			
(+) vs. (-)				
If mdm2 (-)	+- vs --	3.25	(1.25, 8.47)	0.02 s
If mdm2 (+)	++ vs -+	0.2	(0.02, 2.11)	0.18
mdm2				
(+) vs. (-)				
If p53 (-)	-+ vs --	20.02	(1.81, 221.72)	0.01 s
If p53 (+)	++ vs +-	1.23	(0.42, 3.63)	0.7

	p53	mdm2			
24	-	-	1	Ref	
1	-	+	20.02	(1.81, 221.72)	0.01 s
34	+	-	3.25	(1.25, 8.47)	0.02 s
7	+	+	4	(1.01, 15.94)	0.05 s

Control variables:				
Stage	Ta			
	T1	1.61	(0.51, 5.07)	0.41
	TIS	6.73	(1.19, 38.23)	0.03 s
CIS	yes vs. no	6.97	(1.77, 27.40)	0.01 h
Chemotherapy	yes vs. no	0.26	(0.07, 0.94)	0.04 s

Significance tests:	LR Chi-square	df	p-value
Model: LR Chi-square, df, p-value	18.15	7df	0.01 s
LR test of significance:			
Of tumor markers	9.15	3df	0.03 s
vs previous model	3.14	1df	0.03 s

b=borderline significant at .10 level (<.10,>=.05); s=significant at .05 level (<.05,>=.01); h=highly significant at .01 level (<.01);p=p53, m=mdm2, RR=rate ratio, df=degrees of freedom, CI=confidence intervals, (-)=negative, (+)=positive

Table 6: Modelling a contrast : the protective effect of (-) p53 and (-) mdm2 vs. all other combinations for sample analyzed.

	p53, mdm2 and interaction			Contrast		
	RR	95% CI	Wald p-value	RR	95% CI	Wald p-value
Tumor markers:						
p53 mdm2						
24 - -	1	Ref		1	Ref	
1 - +	20.02	(1.81, 221.72)	0.01 s			
34 + -	3.25	(1.25, 8.47)	0.02 s	3.31	(1.29, 8.49)	0.01 s
7 + +	4	(1.01, 15.94)	0.05 s			
				Expressed as (-) p53 and (-) mdm2 vs. other:		
				0.3	(0.12, 0.78)	0.01 s
Control variables:						
Stage						
Ta	1	Ref		1	Ref	
T1	1.61	(0.51, 5.07)	0.41	1.67	(0.55, 5.04)	0.37
TIS	6.73	(1.19, 38.23)	0.03 s	6.77	(1.22, 37.65)	0.03 s
CIS						
yes vs. no	6.97	(1.77, 27.40)	0.01 h	5.73	(1.57, 20.92)	0.01 h
Chemo.						
yes vs. no	0.26	(0.07, 0.94)	0.04 s	0.03	(0.09, 1.02)	0.05 b
Significance tests:	LR Chi-sq	df	p-value	LR Chi-sq	df	p-value
	18.15	7df	0.01 s	16.3	5df	0.01 h
LR test of significance:						
Of tumor markers	9.15	3df	0.03 s	7.3	1df	p<.01 h
Of goodness of fit of contrast				1.85	2df	0.4

b=borderline significance at .10 level (<.10, >=.05); s=significant at .05 level (<.05, >=.01); h=highly significant at .01 level; p=p53, m=mdm2, RR=rate ratio, df=degrees of freedom, CI=confidence intervals, (-)=negative, (+)=positive

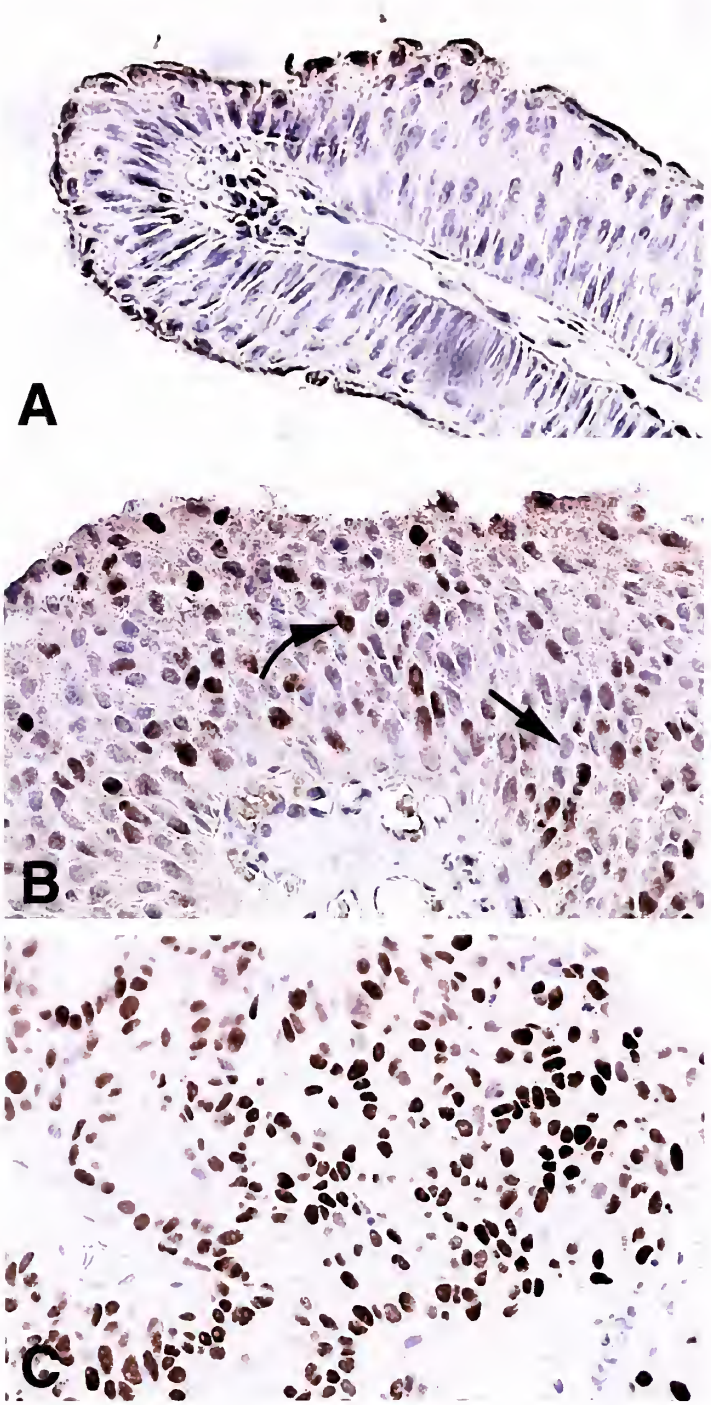


Figure 1. Immunohistochemical detection of p53 protein in the nuclei of transitional-cell carcinomas of the bladder with the anti-p53 monoclonal antibody DO7. **Panel A:** No detectable nuclear staining seen. **Panel B:** Heterogeneous nuclear reactivity is seen in 10 to 49 percent of tumor cells (straight arrow indicates no nuclear staining,curved arrow indicates positively stained nuclei). **Panel C:** Intense homogeneous nuclear reactivity is seen in 50 to 100 percent of tumor cells (all panels x200).

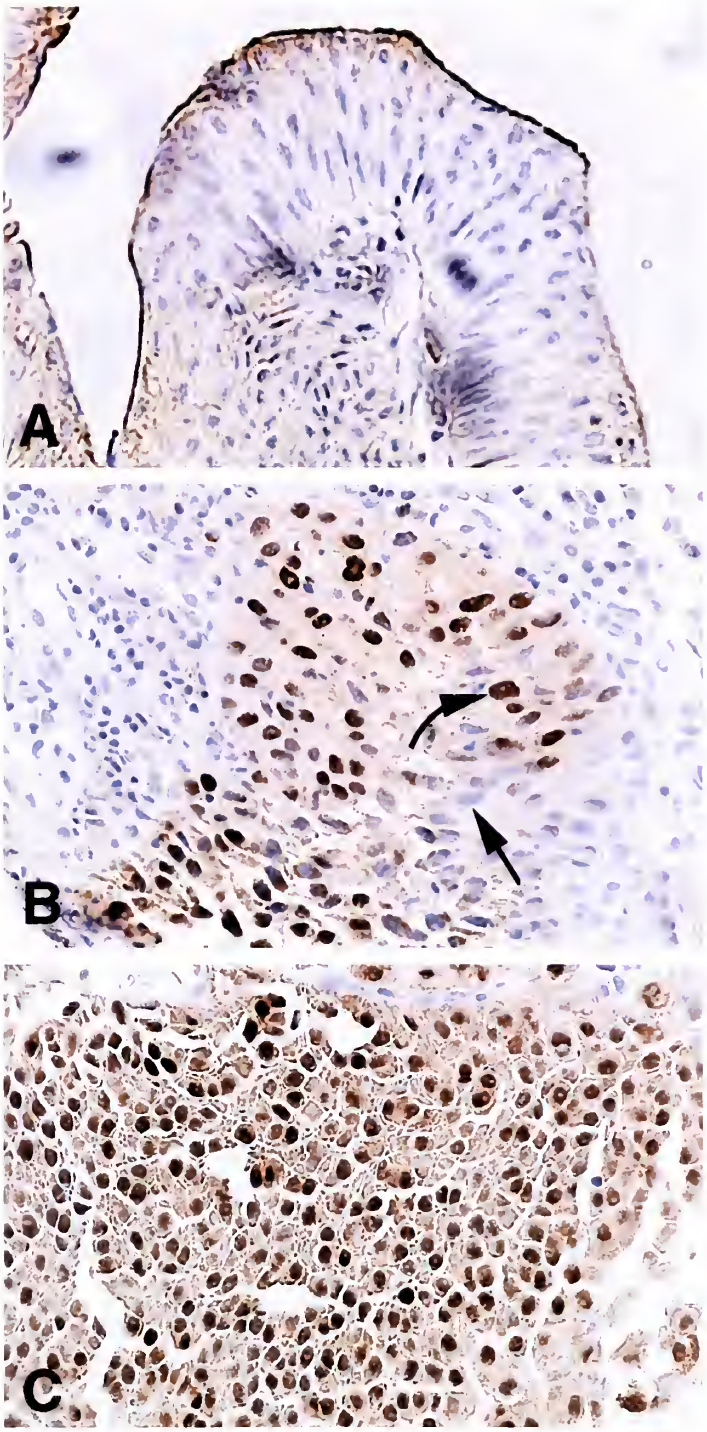


Figure 2. Immunohistochemical detection of MDM2 protein in the nuclei of transitional-cell carcinomas of the bladder with the anti-MDM2 monoclonal antibody IF2. **Panel A:** No detectable nuclear staining seen. **Panel B:** Heterogeneous nuclear reactivity is seen in 10 to 49 percent of tumor cells (straight arrow indicates no nuclear staining,curved arrow indicates positively stained nuclei). **Panel C:** Intense homogeneous nuclear reactivity is seen in 50 to 100 percent of tumor cells (all panels x200).

Discussion

We proposed to analyze the expression of two tumor markers, p53 and mdm2, in patients with superficial bladder cancer. We sought to determine if alterations in the expression of these genes are of any clinical significance in terms of predicting recurrence of bladder tumors. 41 (62%) of the 66 superficial tumors analyzed were positive for overexpression of the p53 protein. 8 (12%) of the 66 superficial tumors analyzed were positive for overexpression of the mdm2 protein. Previous studies of p53 expression in superficial tumors utilizing immunohistochemical (IHC) techniques have not yielded such a high percentage of p53 positive tumors. A study by Esrig et al., found 22% of superficial bladder tumors (Pa, Pis and P1) to be positive for overexpression of the p53 protein[54]. A previous study by Esrig et al., found 33% of superficial tumors (Pa and P1) to be positive for overexpression of the p53 protein[36]. Lipponen found 25% of superficial tumors (Pa and P1) to be positive for overexpression of the p53 protein[55]. A number of explanations can account for our higher percentage of p53 positive superficial tumors. Immunohistochemical findings have already been shown to have a high correlation with molecular studies such as direct sequencing of PCR amplified DNA fragments and single strand conformational polymorphism analysis (SSCP)[36]. Previous studies have shown overexpression of p53 protein in IHC to be highly specific (90%) for mutations in the p53 gene[60]. However, false positives and false negatives still persist. False positives refer to cases in which there was overexpression of p53 protein via IHC analysis but

no detectable abnormality in the p53 gene via molecular genetic analysis. False positives may result from the increased expression of wild-type (no genetic abnormalities) p53 protein. Barnes et al., described an alteration in the promoter region of a normal p53 gene which lead to the overexpression of wild-type protein in a family with breast cancer syndrome[61]. Exposure of cells to DNA damaging elements such as UV light, gamma irradiation and chemotherapeutic agents also results in an increase in the cellular levels of wild-type p53[28,29]. P53 has been shown to form oligomeric protein complexes with a number of viral and cellular proteins[19]. Stabilization of the wild-type protein through complex formation with viral and cellular proteins can result in nuclear accumulation of p53 protein and subsequent false positive detection via IHC techniques. An important question to consider in these studies is how does stabilization of the p53 protein through complex formation with cellular and viral proteins effect the overall function of the p53 protein. A number of independent investigators have shown that p53 loses its transactivation capability when bound to specific viral proteins[38,39,40,42]. False positives may also be the result of inadequate molecular analysis of the p53 gene. The majority of mutations involving the p53 gene are found within exons 5-8 (evolutionarily highly conserved regions) and as a result these regions are the ones analyzed for genetic mutations. However, it is feasible that alterations found via IHC are actually the result of genetic mutations outside of the evolutionarily conserved domains. Lastly, false positives may be due to the insensitive nature of genetic molecular analysis. The sensitivity of genetic molecular analysis depends upon the ratio of genetically mutated DNA to that of normal wild-type DNA. Therefore if the tissue under

analysis has a low ratio of tumorgenic to normal tissue, molecular genetic methods may yield falsely negative results. Sidransky et al., have shown that when the ratio of cells containing mutations is low molecular methods are unable to detect such mutations[62].

False negatives refer to cases in which there was no detectable overexpression of p53 protein via IHC analysis, however, molecular genetic analysis revealed mutations in the p53 gene. False negatives may be the result of specific types of mutations in the p53 gene. For example, a point mutation may code for a stop codon and truncate the protein product making nuclear accumulation and IHC detection impossible. Codon 196 codes for the nuclear localization domain of the protein which is at amino acids 316-325[23]. Mutations that involve the nuclear localization domain of the gene may inhibit the ability of p53 protein to enter the nucleus and thus effect its detection by IHC methods. Certain gross deletions may result in eradication of any p53 protein[48]. Wild-type p53 protein has a relatively short half-life of 5 to 40 minutes[36]. Detection of alterations in the p53 gene by IHC is based on the premise that the majority of mutations involving the p53 gene result in a conformational change of protein structure and subsequent stabilization of the protein. The stabilized protein has an increased half-life of several hours[37]. False negatives may be the result of missense point mutations that do not result in a protein with increased stability and half-life[60]. As discussed above, there are a number of viral and cellular proteins that form complexes with the p53 protein. False negatives may be the result of a complex formation that conceals the binding site (epitope) of the antibody being used in IHC. In a similar fashion, genetic

mutations involving epitopes for particular antibodies may effect their ability to detect accumulated p53 protein via IHC. For example a mutation in exon 5 of the p53 gene may lead to a conformational change in the corresponding portion of the p53 protein. As a result, antibodies that are specific for that portion of the protein will no longer effectively recognize the mutated epitope.

Results of IHC are extremely dependent upon the technical conditions under which they are performed. Factors such as the primary antibody, antigen retrieval methods, and fixatives used in the processing of tissues can all effect the results of IHC. In our study, staining for overexpression of the p53 protein was done with the mouse monoclonal antibody DO7. Most previous studies evaluating overexpression of p53 protein used the mouse monoclonal antibody PAb1801. Data implicating DO7 as a much more sensitive antibody in the detection of overexpressed p53 protein may explain our findings of an increased percentage of superficial tumors positive for p53. Baas et al., undertook a study evaluating six antibodies for IHC detection of p53 in archival colorectal neoplasms[60]. The p53 gene mutational status of all tumors was previously determined through polymerase chain reaction and double-stranded DNA sequencing of the products. Both of the antibodies DO7 and 1801 were included in this study. Baas et al., found that of the six antibodies studied, DO7 in combination with Target Unmasking Fluid (TUF) used for antigen retrieval was the most sensitive and specific in immunostaining of p53[60].

Antigen retrieval methods also appear to affect the results of IHC. Our study utilized an antigen retrieval method similar to that of the TUF method. We incubated tissues in a citrate buffer for a total of ten minutes in a standard microwave

unit. The TUF method consists of immersing the tissue in a solution and heating for ten minutes at 90°C in a temperature probe-controlled microwave oven. Prior studies of Esrig et al., and Lipponen which determined expression of p53 in superficial tumors had not utilized any method of antigen retrieval[36,54,55]. Our use of the most sensitive and specific antibody available coupled with antigen retrieval methods can explain our findings of superficial tumors with a higher percentage of positive staining for p53.

In our study, we found there to be an increased risk of recurrence with superficial bladder cancer for patients who had any aberration in the status of their tumor markers, p53 and mdm2. Consistent with the literature, we found that the presence of CIS had an increased risk of recurrence[9]. Also consistent with the literature, we found that alterations in the tumor marker p53 had an increased risk of recurrence[54]. New findings in our study indicate that alterations in the tumor marker mdm2 may aid in the ability of p53 to predict bladder cancer recurrence. This is described in our model of p53, mdm2, and their interaction. Mdm2 is a cellular protein which has the capability of binding and inactivating wild-type p53[46]. A scenario could exist in which there is normal expression of p53 however its function may be altered due to binding with mdm2. One can also describe a scenario in which expression of p53 and mdm2 are both altered. If binding of p53 by mdm2 leads to a stable complex formation one would expect that overexpression of both would be detected by IHC methods. It is the stable protein complex formation of p53 (with an increased half-life) that determines its overexpression detected by IHC. For the one subject that was p53 negative and mdm2 positive we found there

to be a high risk of recurrence. This correlates with our proposed hypothesis that mdm2 may be functioning to inactivate wild-type p53. However, this data should be interpreted cautiously because it is based only upon one subject. We found that mdm2 as a tumor marker significantly added to our model of p53 and its ability to predict bladder cancer recurrence. This was demonstrated through the likelihood ratio test of significance of our tumor markers. When examining both tumor markers p53 and mdm2 we found that any abnormality in their expression correlated with an increased risk of recurrence that was 3.31 times that of normal tumor marker expression.

Our findings have significant clinical implications. The ability to predict which superficial tumors are more likely to recur and progress will have an enormous impact on therapeutic options. This study may have implications in the surgical treatment of superficially invasive disease. For example, patients having superficially invasive disease and alterations in either of the tumor markers, p53 and/or mdm2, might consider more aggressive treatment options, such as cystectomy, based on the findings that they are more likely to recur, progress and possibly die of their disease. Conversely, patients with normal expression of tumor markers and locally advanced disease might consider more conservative measures of treatment based on the lower likelihood of disease recurrence and progression. Such conservative measures might include systemic chemotherapy and radiation treatment or partial cystectomy and subsequent intravesical chemotherapy. P53 status also has implications on current chemotherapeutic options. As stated earlier, p53 is thought to be involved in apoptosis. As a result p53 negative tumors (wild-

type) are thought to be more sensitive to chemotherapy because the pathway to programmed cell death remains intact.

This study suffered from a relatively small patient pool. Future studies may wish to address the same questions posed here but in a larger patient population. IHC has allowed for the practical evaluation of genomic alterations involved in tumorgenisis. However, future areas of study may wish to focus on the development of a test that could assess the function of molecules involved in tumorigenesis like p53 and mdm2.

Bibliography

1. Sidransky, D., Von Eschenbach, A., Tsai, Y.C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S.R., Frost, P., and Vogelstein, B. Identification of p53 Gene Mutations in Bladder Cancers and Urine Samples. Science, 252: 706-709, 1991.
2. Isselbacher, K.J., Braunwald, E., Wilson, J.D., Martin, J.B., Fauci, A.S., Kasper, D.L., eds. 1994. Harrison's Principles of Internal Medicine 13th ed. McGraw-Hill, Inc.
3. Walsh, P.C., Retik, A.B., Stamey, T.A., Vaughan, E.D., eds. 1992. Campbell's Urology 6th ed. W.B. Saunders Company.
4. Althausen, A. F., Prout, G. R., Jr., and Daly, J. J.: Noninvasive papillary carcinoma of the bladder associated with carcinoma in situ. J. Urol., 116:575, 1976.
5. Fitzpatrick, J. M., West, A. B., Butler, M. R., et al.: Superficial bladder tumors (stage pTa, grades 1 and 2): The importance of recurrence pattern following initial resection. J. Urol., 135:920, 1986.
6. Gilbert, H. A., Logan, J. L., Lagan, A. R., et al.: The natural history of papillary transitional cell carcinoma of the bladder and its treatment in an unselected population on the basis of histologic grading. J. Urol., 119:486, 1978.

7. Malmstrom, P. U., Busch, C., and Norlen, B. J.: Recurrence, progression and survival in bladder cancer: A retrospective analysis of 232 patients with greater than or equal to 5-year follow-up. Scand. J. Urol. Nephrol., 21:185, 1987.

8. Tanagho, E. A. and McAninch, J. W., eds. 1995. Smith's General Urology 14th ed. Norwalk, Connecticut: Appleton & Lange.

9. Lamm, D. L., ed. The Urologic Clinics of North America: Superficial Bladder Cancer., 19:3. August, 1992.

10. Marshall, C. J., Tumor Suppressor Genes. Cell, 64: 313-326, 1991.

11. Bartek, J., Bartkova, B., Vojtesek, Z., et al.: Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene, 6:1699-1703, 1991.

12. Cavenee, W. K., Hastie, N. D., and Standbridge, E. J., eds. (1989). "Current Communications in Molecular Biology: Recessive Oncogenes and Tumor Suppression." Cold Spring Harbor Press, Cold Spring Harbor, New York.

13. Lee, J. H., et al. (1989). Proc. Am. Assoc. Cancer Res. 30, 442.

14. Atkin, N. B., and Baker, M. C.: Chromosome 17p loss in carcinoma of the cervix uterine. Cancer Genetics and Cytogenetics 37: 229-233, 1989.

15. Yano, T., Linehan, M., Anglard, P., Lerman, M. I., Daniel, L. N., Stein, C. A., Robertson, C. N., LaRocca, R., Zbar, B.: Genetic changes in human adrenocortical carcinomas. Journal of the National Cancer Institute 81: 518-523, 1989.

16. Tsai, C. M., Gazdar, A. F., Venzon, D. J., Steinberg, S. M., Dedrick, R. L., Mulshine, J. L., Kramer, B. S.: Lack of in vitro synergy between etoposide and cis-diammine-dichloroplatinum (II). Cancer Research 49: 2390-2397, 1989.

17. Fearon, E. R., Hamilton, S. R., and Vogelstein, B.: Clonal analysis of human colorectal tumors. Science 238: 193-198, 1987.

18. Monpezat, J. P. H., Delattre, O., Bernard A., Grunwald, D., Remvikos, Y., Muleris, M., Salmon, R. J., Frelat, G., Dutrillaux, B., Thomas, G.: Loss of alleles on chromosome 18 and on the short arm of chromosome17 in polyploid colorectal carcinomas. Int. J. Cancer 41: 404-408, 1988.

19. Furihata, M., Sonobe, H., and Ohtsuki, Y., The aberrant p53 protein (Review). International Journal of Oncology 6:1209-1226, 1995.

20. Hollstein, M., Sidransky, D., Vogelstein, B., et al. P53 mutations in human cancers. Science 253: 49-53, 1991.

21. Caron de Fromentel, C., and Soussi, T., TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosome Cancer 4: 1-15, 1992.

22. Funk, W. D., Pak, D. T., Karas, R. H., et al.: A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol 12: 2866-2871,1992.

23. Vogelstein, B., and Kinzler, K. W., p53 function and dysfunction. Cell, 70: 523-526, 1992.

24. Mack, D. H., Vartikar, J., Pipas, J. M., et al.: Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. Nature 363: 281-283, 1993.

25. Finlay, C. A., Hinds, P. W., and Levine, A. J. The p53 proto-oncogene can act as a suppressor of transformation. Cell 57: 1083-1093, 1989.

26. Eliyahu, D., Michalovitz, D., Eliyahu, S., et al.: Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA 86: 8763-8767, 1989.

27. Chen, P. L., Chen, Y., Bookstein, R., et al.: Genetic mechanisms of tumor suppression by the human p53 gene. Science 250: 1576-1580, 1990.

28. Maltzman, W., and Czyzyk, L., UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell Biol. 4: 1689-1694, 1984.

29. Kastan, M. B., Onyekwere, O., Sidransky, D., et al.: Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311, 1991.

30. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., et al.: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 89: 7491-7495, 1992.

31. Yonish-Rouach, E., Resnitzky, D., Lotem, J., et al.: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352: 345-347, 1991.

32. Shaw, P., Bovey, R., Tardy, S., et al.: Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA 89: 4495-4499, 1992.

33. El-Deiry, W. S., Tokino, T., Velculescu, V. E., et al.: WAF-1, a potential mediator of p53 tumor suppression. Cell 75: 817-825, 1993.

34. Hayashi, K.: PCR-SSCP: a simple sensitive method for detection of mutations in the genomic DNA (Review). PCD Methods Appl. 1:34-38, 1991.

35. Suzuki, Y., Sekiya, T., and Hayashi, K.: Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. Anal. Biochem. 192: 82-84, 1991.

36. Esrig, D., Spruck, C. H. III., Nichols, P. W., et al.: p53 nuclear protein accumulation correlates with mutation in the p53 gene, tumor grade, and stage in bladder cancer. American Journal of Pathology 143: 1389-1397, 1993.

37. Finlay, C. A., Hinds, P. W., Tan, T. H., Eliyahu, D., Oren, M., Levine, M. J.: Activating mutations for transformation by p53 produce a gene product that forms an hsc 70-p53 complex with an altered half life. Mol. Cell Biol. 8: 531-539, 1988.

38. Lane, D. P., and Crawford, L.V.: T-antigen is bound to host protein in SV 40-transformed cells. Nature 278: 261-263, 1979.

39. Linzer, D. I. H., and Levine, A. J.: Characterization of a 54 K dalton cellular SV 40 tumor antigen present in SV 40 transformed cells and uninfected embryonal carcinoma cells. Cell 17: 43-52, 1979.

40. Werness, B. A., Levine, A. J., and Howley, P.: Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248: 76-79, 1990.

41. Szekely, L., Selivanova, G., Magnusson, K. P., et al.: EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. Proc. Natl. Acad. Sci. USA 90: 5455-5459, 1993.

42. Sarnow, P., Ho, Y. S., Williams, J., et al.: Adenovirus E1b-58kd tumor antigen and SV 40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell 28: 387-394,1982.

43. Feitelson, M. A., Zhu, M., Duan, L. X., et al.: Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene 8: 1109-1117, 1993.

44. Symonds, H., Chen, J., and Van Dyke, T.: Complex formation between the lymphotropic papovavirus large tumor antigen and the tumor suppressor protein p53. J. Virol. 65: 5417-5424, 1991.

45. Levine, A. J., Momand, J., and Finlay, C. A.: The p53 tumor suppressor gene. Nature 351: 453-456, 1991.

46. Rogel, A., Popliker, M., Webb, C. G., et al.: p53 cellular tumor antigen: Analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol. Cell Biol. 5: 2851-2855, 1985.

47. Momand, J., Zambetti, G. P., Olson, D. C., et al.: The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237-1245, 1992.

48. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., Vogelstein, B.: Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 358: 80-83, 1992.

49. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., et al.: Oncoprotein mdm2 conceals the activation domain of tumor suppressor p53. Nature 362: 857-860, 1993.

50. Habuchi, T., Kinoshita, H., Yamada, H., et al.: Oncogene amplification in urothelial cancers with p53 gene mutation or mdm2 amplification. Journal of the National Cancer Institute 86: 1331-1335, 1994.

51. Cahilly-Snyder, L., Yang-Feng, T., Francke, U., and George, D. L.: Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somatic Cell Mol. Genet. 13: 235-244, 1987.

52. Fakharzadeh, S. S., Trusko, S. P., and George, D. L.: Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J. 10: 1565-1569, 1991.

53. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J.: The p53-mdm-2 autoregulatory feedback loop. Genes & Development 7: 1126-1132, 1993.

54. Esrig, D., Elmajian, D., Groshen, S., Freeman, J. A., Stein, J. P., Chen, S., Nichols, P. W., Skinner, D. G., Jones, P. A., and Cote, R. J.: Accumulation of nuclear p53 and tumor progression in bladder cancer. The New England Journal of Medicine 331: 1259-1264, 1994.

55. Lipponen, P. K.: Over-expression of p53 nuclear oncoprotein in transitional-cell bladder cancer and its prognostic value. Int. J. Cancer 53: 365-370, 1993.

56. Sarkis, A. S., Dalbagni, G., Cordon-Cardo, C., Zhang, Z., Sheinfeld, J., Fair, W. R., Herr, H. W., and Reuter, V. E.: Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: A marker for disease progression. Journal of the National Cancer Institute 85: 53-59, 1993.

57. Sarkis, A. S., Dalbagni, G., Cordon-Cardo, C., Melamed, J., Zhang, Z., Sheinfeld, J., Fair, W. R., Herr, H. W., and Reuter, V. E.: Association of p53 nuclear

overexpression and tumor progression in carcinoma in situ of the bladder. The Journal of Urology 152: 388-392, 1994.

58. Sarkis, A. S., Bajorin, D. F., Reuter, V. E., Herr, H. W., Netto, G., Zhang, Z., Schultz, P. K., Cordon-Cardo, C., and Scher, H. I.: Prognostic value of p53 nuclear overexpression in patients with invasive bladder cancer treated with neoadjuvant MVAC. Journal of Clinical Oncology 13: 1384-1390, 1995.

59. Leach, F. S., Tokino, T., Meltzer, P., Burrell, M., Oliner, J. D., Smith, S., Hill, D. E., Sidransky, D., Kinzler, K. W., and Vogelstein, B.: p53 mutation and mdm2 amplification in human soft tissue sarcomas. Cancer Research 53: 2231-2234, 1993.

60. Baas, I. O., Mulder, J. R., Offerhaus, G. J. A., Vogelstein, B., and Hamilton, S. R.: An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. Journal of Pathology 172: 5-12, 1994.

61. Barnes, D. M., Hanby, A. M., Gillett, C. E., et al.: Abnormal expression of wild-type p53 protein in normal cells of a cancer family patient. Lancet 340: 259-263, 1992.

62. Sidransky, D., Mikkelsen, T., Schwechheimer, K., Rosenblum, M. L., Cavanee, W., Vogelstein, B.: Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature 355: 846-847, 1992.

63. Cairns, P., Proctor, A. J., Knowles, M. A.: Loss of heterozygosity at the RB locus is frequent and correlates with muscle invasion in bladder carcinoma. Oncogene 6: 2305-2309, 1991.

64. Xu, H., Cairns, P., Hu, S., Knowles, M. A., Benedict, W. F.: Loss of RB protein expression in primary bladder cancer correlates with loss of heterozygosity at the RB locus and tumor progression. Int. J. Cancer 53: 781-784, 1993.

65. Cordon-Cardo, C., Wartinger, D., Petrylak, D., Dalbagni, G., Fair, W. R., Fuks, Z.,Reuter, V. E.: Altered expression of the retinoblastoma gene product: Prognostic indicator in bladder cancer. Journal of the National Cancer Institute 84: 1251-1256, 1992.

66. Logothetis, C. J., Xu, H., Ro, J. Y., Hu, S., Sahin, A., Ordonez, N., Benedict, W. F.: Altered expression of retinoblastoma protein and known prognostic variables in locally advanced bladder cancer. Journal of the National Cancer Institute 84: 1256-1261, 1992.

67. Presti, J. C., Reuter, V. E., Galan, T., Fair, W. R., Cordon-Cardo, C.: Molecular genetic alterations in superficial and locally advanced human bladder cancer. Cancer Research 51: 5405-5409, 1991.

68. Hirata, Y., Orth, D. N.: Epidermal growth factor (urogastrone) in human fluids: size heterogeneity. J. Clin. Endocrinol. Metab., 48: 673-679, 1979.

69. Messing, E. M., Reznikoff, C. A.: Normal and malignant human urothelium: *in vitro* effects of epidermal growth factor. Cancer Research 47: 2230-2235, 1987.

70. Dubeau, L., Jones, P. A.: Growth of normal and neoplastic urothelium and response to epidermal growth factor in a defined serum-free medium. Cancer Research 47: 2107-2112, 1987.

71. Messing, E. M.: Clinical implications of the expression of epidermal growth factor receptors in human transitional cell carcinoma. Cancer Research 50: 2530-2537, 1990.

72. Berger, M. S., Greenfield, C., Gullick, W. J., Haley, J., Downward, J., Neal, D. E., Harris, A. L., Waterfield, M. D.: Evaluation of epidermal growth factor receptors in bladder tumours. British Journal of Cancer 56: 533-537, 1987.

73. Wright, C., Mellon, K., Johnston, P., Lane, D. P., Harris, A. L., Horne, C. H. W., Neal, D. E.: Expression of mutant p53, c-erbB-2 and the epidermal growth factor receptor in transitional cell carcinoma of the human urinary bladder. British Journal of Cancer 63: 967-970, 1991.

74. Cartwright, C. A., Simantov, R., Cowan, W. M., Hunter, T., Eckhart, W.: $pp60^{c\text{-}src}$ expression in the developing rat brain. Proc. Natl. Acad. Sci. USA 85: 3348-3352, 1988.

75. Brugge, J. S., Cotton, P. C., Queral, A. E., Barrett, J. N., Keane, R. W.: Neurons express high levels of a structurally modified, activated form of $pp60^{c\text{-}src}$. Nature (Lond.) 316: 554-557, 1985.

76. Brugge, J. S., Cotton, P. C., Lustig, A., Yonemoto, W., Lipsich, L., Coussens, P., Barrett, J. N., Nonner, D., Keane, R. W.: Characterization of the altered form of the c-src gene product in neuronal cells. Genes Dev., 1: 287-296, 1987.

77. Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V., Rosen, N.: Activation of $pp60^{c\text{-}src}$ protein kinase activity in human colon carcinoma. Proc. Natl. Acad. Sci. USA 84: 2251-2255, 1987.

78. Cartwright, C. A., Meisler, A. I., Eckhart, W.: Activation of the pp60$^{c\text{-}src}$ protein kinase is an early event in colonic carcinogenesis. Proc. Natl. Acad. Sci. USA 87: 558-562, 1990.

79. Fanning, P., Bulovas, K., Saini, K. S., Libertino, J. A., Joyce, A. D., Summerhayes, I. C.: Elevated expression of pp60$^{c\text{-}src}$ in low grade human bladder carcinomas. Cancer Research 52: 1457-1462, 1992.

80. Land, H., Paroda, G., Weinberg, R.: Cellular oncogenes and multistep carcinogenesis. Science 222: 771, 1983.

81. Vogelzang, N. J., Scardino, P. T., Shipley, W. U., Coffey, D. S., eds. 1996. Comprehensive Textbook of Genitourinary Oncology. Williams & Wilkins.

82. Ooi, A., Herz, F., Ii, S., Cordon-Cardo, C., Fradet, Y., Mayall, B. H., Maker, N. I. H.: Ha-ras codon 12 mutation in papillary tumors of the urinary bladder. A retrospective study. Int. J. Oncol. 4: 85-90, 1994.

83. Viola, M. V., Fromowitz, F., Oravez, S., Deb, S., Schlom, J.: ras oncogene p21 expression is increased in premalignant lesions and high grade bladder carcinoma. Journal of Experimental Medicine 161: 1213-1218, 1985.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE